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Alterations in Peptidoglycan Precursors and Vancomycin Susceptibility in Tn*917* Insertion Mutants of *Enterococcus faecalis* 221

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Derivatives of the highly vancomycin-resistant *Enterococcus faecalis* strain 221 (MIC, 1,024 μg/ml) harboring Tn*917* insertions in *vanR*, *vanH*, and *vanA* were compared with the parent strain and the susceptible plasmid-free strain JH2-2 (MIC, 2 μg/ml). Cytoplasmic pools of UDP-*N*-acetyl-muramyl-peptide precursors of strain 221 contained the depsipeptide-terminating precursor as well as elevated levels of both the tripeptide and tetrapeptide precursors. Insertional inactivation of *vanR* resulted in the loss of carboxypeptidase activity, full susceptibility to vancomycin, and precursor pools similar to those of JH2-2. For the *vanA* insertional mutant the MBC of vancomycin was fourfold higher than that for JH2-2, and the mutant had increased levels of tripeptide and tetrapeptide precursors compared with those for JH2-2. The *vanH* insertional mutant showed elevated levels of these precursors, as well as a small amount of depsipeptide, and both the MIC and the MBC of vancomycin were increased compared with those for JH2-2. These findings suggest that DD-carboxypeptidase activity, under the control of *vanR*, results in increased pools of both tripeptide and tetrapeptide precursors, which may contribute to survival in the presence of vancomycin.

High-level glycopeptide resistance in *Enterococcus faecalis* and *Enterococcus faecium* is associated with a cluster of genes which in some strains resides on a transposon (5). Four genes in the *van* cluster are required for high-level vancomycin resistance: the regulatory gene *vanR* (4, 13), the structural genes *vanA* and *vanH*, and a gene of unknown function, *vanX* (5, 8). *vanH* encodes an α-keto reductase that is able to produce D-lactate from pyruvate (8), and *vanA* encodes a ligase that synthesizes the depsipeptide D-alanyl–D-lactate (7). This depsipeptide is incorporated at the terminus of the cytoplasmic precursor of peptidoglycan in place of the normal dipeptide D-alanyl–D-alanine (D-Ala–D-Ala), preventing vancomycin binding (1, 15, 17).

In addition to the genes required for high-level expression of resistance, the accessory gene *vanY* has been identified in isolates carrying the *van* gene cluster (3). *vanY* encodes a membrane-associated DD-carboxypeptidase that has been shown in vitro to hydrolyze the normal pentapeptide precursor, as well as the dipeptide D-Ala–D-Ala and the depsipeptide D-Ala–D-lactate (3, 21). Carboxypeptidase activity has been identified in all resistant strains thus far, and the level of activity appears to correlate with the level of resistance (2, 11, 21), suggesting that the carboxypeptidase may play a role in cell wall synthesis in these strains.

In the present study, analysis of cytoplasmic precursors from the vancomycin-resistant strain *E. faecalis* 221 and transposon mutants was used to assess the roles of the required and accessory genes in production of altered peptidoglycan precursors and survival in the presence of vancomycin. The findings suggest that although production of depsipeptide-terminating precursors is required for high-level resistance, the elevated amounts of UDP-*N*-acetyl-muramyl-tripeptide and -tetrapeptide precursors in resistant cells may augment the ability of these organisms to survive in the presence of vancomycin.

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MATERIALS AND METHODS

Bacterial strains. *E. faecalis* strains used are listed in Table 1. Plasmid pHKK100 is a conjugative vancomycin resistance plasmid isolated from wild-type *E. faecium* 228 (14). *E. faecalis* was grown in brain heart infusion (Difco, Detroit, Mich.) unless otherwise specified. *Staphylococcus aureus* 209P was grown in tryptic soy broth (Difco).

Susceptibility testing. MICs were determined by broth macrodilution in Mueller-Hinton broth (Difco). MBCs were determined by the macrodilution method (19).

Transposon mutagenesis. Tn*917* mutagenesis of pHKK100, done with the temperature-sensitive delivery vector pTV1Ts, was performed as previously described (13). Vancomycin-susceptible mutants were identified by replica plating onto tryptic soy agar containing 100 μg of vancomycin per ml. Plasmid DNA was purified by the method of Weaver and Clewell (20). Sites of Tn*917* insertion were determined by restriction endonuclease digestion of plasmid DNA as recommended by the manufacturer (GIBCO BRL, Gaithersburg, Md.).

Analysis of peptidoglycan precursors. Peptidoglycan precursors were prepared by a modification of a previously described method (15). Briefly, cultures were grown in brain heart infusion to mid-logarithmic phase, chilled rapidly, harvested by centrifugation, and extracted with cold trichloroacetic acid (final concentration, 5%) for 30 min. After centrifugation, the supernatant fluid was separated by gel filtration (Sephadex G-25; Pharmacia, Alameda, Calif.), with elution with water. Hexosamine-containing fractions were identified by the assay of Ghuysen et al. (10), pooled, and lyophilized. Reverse-phase high-performance liquid chromatography (HPLC) separation was performed with a μBondapak C_{18} column (3.9 by 300 mm) (Waters, Milford, Mass.), with isocratic elution with 0.05 M ammonium formate, pH 4.75. Precursors were identified by comparison with UDP-*N*-acetyl-muramyl-pentapeptide and -tripeptide prepared from *S. aureus* 209P, by amino acid analysis, and by mass spectrometry as previously described (15). In order to avoid confounding effects of different concen-

TABLE 1. Testing of susceptibility of *E. faecalis* to vancomycin and determination of UDP-*N*-acetyl-muramyl-peptide precursor pool concentrations in *E. faecalis*

Strain	Tn*917* site	MIC/MBC[a]	Concn of vancomycin (μg/ml)[b]	Concn of cytoplasmic precursor pool[c] (nmol/10^{11} cells)			
				Tri	Tetra	Penta	Depsi
JH2-2		2/64	0.5	12	6	42	<2
221 [JH2-2(pHKK100)]		1,024/>1,024	0	36	18	74	14
		1,024/>1,024	100	37	33	10	104
789 [JH2-2(pHKK100::Tn*917*)]	*vanR*	2/64	0.5	15	2	42	<2
747 [JH2-2(pHKK100::Tn*917*)]	*vanA*	2/256	0.5	30	22	37	<2
728 [JH2-2(pHKK100::Tn*917*)]	*vanH*	32/512	0.5	19	12	35	5

[a] Identical results obtained from three determinations.

[b] Concentration of vancomycin in which cultures were grown for precursor extraction.

[c] Tri, tetra, and penta are UDP-*N*-acetyl-muramyl peptide precursors terminating in tripeptide, tetrapeptide, and pentapeptide, respectively. Depsi is the depsipeptide-terminating precursor UDP-*N*–acetyl–muramyl-L-Ala–D-Glu–L-Lys–D-Ala–D-lactate.

trations of vancomycin on precursor accumulation, 0.5 μg of vancomycin per ml was added to all cultures. This concentration was sufficient to induce resistance in resistant strains, but it did not inhibit growth in susceptible strains. Aliquots of cultures were removed for plating at the time of cell harvest. Results are the means of two to three determinations, expressed as nanomoles per 10^{11} CFU. Pool levels from different preparations showed close agreement (20% or less variation).

Carboxypeptidase assays. Cell membranes were prepared as previously described (16), except that cultures were grown in brain heart infusion. The substrate UDP-*N*-acetyl-muramyl-pentapeptide (1 mM) obtained from *S. aureus* 209P was incubated with purified cell membranes (1 mg/ml) in 10 mM potassium phosphate buffer, pH 8.0, at 37°C. After 60 min, the mixture was heated to 70°C for 3 min and centrifuged to remove debris, and the supernatant was separated by reverse-phase HPLC as described above. Quantities of UDP-*N*-acetyl-muramyl-tripeptide, -tetrapeptide, and -pentapeptide were determined by integration. The addition of Zn^{2+} (5 mM), Mg^{2+} (5 mM), or Triton X-100 (0.1%) did not enhance carboxypeptidase activity.

RESULTS AND DISCUSSION

Table 1 shows susceptibility testing results and cytoplasmic precursor pool concentrations for *E. faecalis* strains grown in various concentrations of vancomycin. The depsipeptide-terminating precursor was detected in small amounts in extracts of cultures of strain 221 grown in the absence of vancomycin, and the amounts were increased with higher concentrations of the antibiotic. Both UDP-*N*-acetyl-muramyl-tripeptide and -tetrapeptide levels were also increased in strain 221, even in the absence of vancomycin.

Although several investigators have noted alterations in the cytoplasmic precursors of vancomycin-resistant enterococci, disparate results concerning the nature of these alterations have been reported. Some investigators have identified accumulation of tetrapeptide precursors in resistant cells (1, 17), consistent with in vivo activity of the carboxypeptidase on the pentapeptide precursor. Others have demonstrated an increase in levels of tripeptide but not tetrapeptide precursors, suggesting that the carboxypeptidase may act primarily to deplete levels of D-Ala–D-Ala, thus diminishing production of the normal pentapeptide precursor (6). The accumulation of tri- and tetrapeptide precursors in *E. faecalis* 221 suggests that the carboxypeptidase may act on both substrates in this strain.

LD-Carboxypeptidase activity resulting in a high proportion of tripeptides in the peptidoglycan has been identified in *E. faecium* (9). To exclude the possibility that tripeptide precursors resulted from the action of an LD-carboxypeptidase on UDP-*N*-acetyl-muramyl-pentapeptide, purified precursor was incubated with strain 221 membranes, and the products were analyzed by reverse-phase HPLC. Pentapeptide was converted to tetrapeptide at the rate of 1.5 nmol/min/mg of membrane protein. No significant amounts of the tripeptide precursor were detected (<0.01 nmol/min/mg).

Strain 789, harboring a Tn*917* insertion in *vanR*, was fully susceptible to vancomycin as determined by the MIC and MBC. No carboxypeptidase activity was detected in this strain (<0.01 nmol/min/mg, similar to that for the susceptible strain JH2-2). Analysis of peptidoglycan precursors of strain 789 showed results similar to those for the wild-type susceptible strain JH2-2.

Carboxypeptidase activity has been shown to be inducible by vancomycin (2, 11, 21), suggesting that *vanY* may be cotranscribed with the other components of the *van* cluster. Analysis of transcriptional fusions by Arthur et al. (4) showed that *vanR* activates a promoter for cotranscription of *vanH*, *vanA*, and *vanX* in response to the presence of vancomycin, but transcription of *vanY* was not studied. The lack of either detectable carboxypeptidase activity or accumulation of tri- and tetrapeptide precursors in strain 789 confirms that *vanR* is required for expression of *vanY*.

The MIC of vancomycin for strain 747, which harbors Tn*917* in *vanA*, was identical to that for the susceptible strain JH2-2, but the MBC was fourfold higher. Strain 747 also had increased pools of both tripeptide and tetrapeptide precursors. It has been suggested that identification of tetrapeptides in precursor pools of resistant cells is an artifact of chemical breakdown of the depsipeptide precursor during preparation (17). The identification of tetrapeptides in the *vanA* mutant, which does not produce depsipeptide precursors, suggests that this is unlikely.

Strain 728, which carries a Tn*917* insertion in *vanH*, was more susceptible to vancomycin than strain 221 but was not fully susceptible. Although analyses of Mueller-Hinton broth obtained from Difco Laboratories did not reveal the presence of lactate, the presence of contaminating small amounts cannot be excluded. However, Arthur et al. have shown that in *E. faecalis* harboring pAT80, a plasmid that carries a portion of the *van* cluster but lacks *vanY*, insertional inactivation of *vanH* results in full susceptibility when this strain is tested in Mueller-Hinton broth (4). The observed difference between *vanH* mutants of pAT80 and pHKK100 suggests a possible role for *vanY*. Carboxypeptidase activity directed preferentially against the normal dipeptide may favor formation of depsipep-

tide precursors even in the presence of very low concentrations of lactate. Alternatively, the observed difference may be related to other genetic elements present on the plasmid or to differences in copy number between pAT80 and pHKK100.

The MBCs of vancomycin for both strain 747 and strain 728 were elevated. Since these strains accumulate tri- and tetrapeptide precursors, it is possible that utilization of these for cell wall synthesis may contribute to survival in the presence of vancomycin. Tri- and tetrapeptide cell wall precursors, lacking the D-Ala–D-Ala terminus, are not bound by vancomycin (18). Although these precursors cannot act as donors in transpeptidation, they may be used in transglycosylation (12). Conceivably, in resistant cells the utilization of such precursors rather than only pentapeptides may allow continued survival in the presence of vancomycin during the induction phase, until adequate pools of depsipeptide precursors are formed. Further investigation will be needed to determine to what extent such precursors are incorporated into peptidoglycan and the effects on peptidoglycan cross-linking.

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